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THE

SCIENTIFIC PROCEEDINGS

OF THE

ROYAL DUBLIN SOCIETY

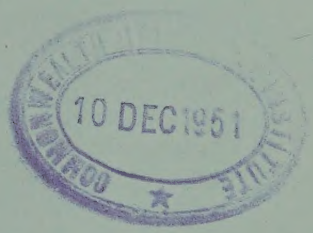
VOL. 25 (N.S.)

No. 22. NOVEMBER, 1951

STUDIES IN INDOLE METABOLISM.

II.—THE ESTIMATION OF INDOXYL.

By JAMES ANTONY DRUM.



DUBLIN :
PUBLISHED BY THE ROYAL DUBLIN SOCIETY,
BALL'S BRIDGE, DUBLIN

1951

Price : One Shilling.

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STUDIES IN INDOLE METABOLISM.
II.—THE ESTIMATION OF INDOXYL.

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JAMES ANTONY DRUM.

[Read March 14. Published November 26, 1951.]

The current view of indole metabolism in the higher animals is that indole, which arises by the bacterial decomposition of tryptophan in the intestine, is absorbed and converted by oxidation, into β-hydroxy indole or indoxyl, a process which takes place mainly in the liver. This indoxyl is then conjugated and excreted in the urine as an ethereal sulphate, potassium indoxyl sulphate, or ' urinary indican.' It is held by some that indoxyl is also excreted as a glycuronide (Neuberg and Schwenk, 1917). On treatment with mineral acid these compounds are decomposed, and the released indoxyl can be estimated.

The earlier methods of indoxyl estimation involved the hydrolysis with strong acid in the presence of an oxidizing agent. The resultant indigo was extracted with chloroform and compared with a standard solution. In some methods, the solution of indigo was titrated with an oxidizing agent, such as NaOBr, the amount required to decolorize the solution being a measure of the amount of indigo present. These methods, crude and quantitatively inadequate by modern standards, have also been criticized by Meikeljohn and Cohen (1941) on the grounds that the color of a solution of indigo extracted from urine may be deeper than that of a saturated solution of indigo.

Jolles (1913) introduced a method for the detection of indoxyl by condensing it with thymol in the presence of an oxidizing agent, and later developed it as a colorimetric method for the estimation of ' indican ' in urine and in blood. The condensation product, a deep violet-coloured dyestuff, is extracted with chloroform and compared with a standard prepared from pure indican. Sharlit (1933) investigated the method and found it to be quantitatively unsatisfactory, and introduced a modification for which he claimed quantitative accuracy and much higher sensitivity. Meikeljohn and Cohen (1941) introduced a photometric modification. In all cases, the standards were prepared from pure indican and thymol.

Tomita (1924) described the detection of indican with ninhydrin. Kumon (1934) developed the test for quantitative estimation. Standards are prepared from pure indican and ninhydrin.

A considerable objection to these methods of estimation, is the use of pure indican in standards, as it is expensive and difficult to obtain. An alternative method for estimation is the hydrolysis of indican in the presence of isatin, forming

indirubin, which may be extracted and compared with indirubin standards. This reaction has several advantages :

(1) It is a condensation reaction requiring no oxidation, which is one of the major sources of error.

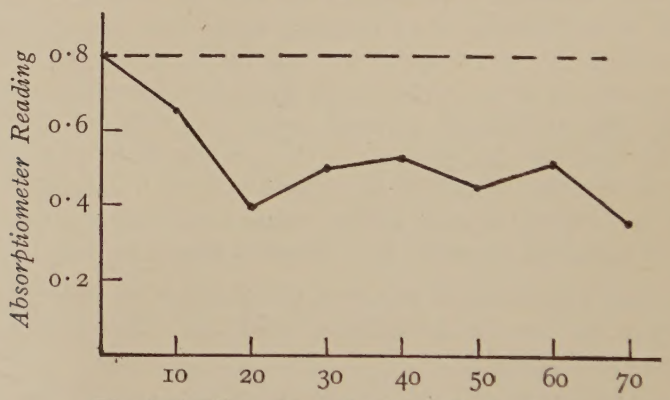
(2) One molecule of indirubin is produced for one molecule of indoxyl.

(3) The standard, indirubin, is easily obtained or prepared.

The possibility of estimating indoxyl by this reaction, discovered by Baeyer (1881), was first suggested by Beijerinck (1899). Bouma (1901) used the reaction for the colorimetric estimation of indican. The indican was hydrolysed with hydrochloric acid in the presence of isatin, and the resultant indirubin extracted with chloroform and compared with a standard or estimated volumetrically by titration with potassium permanganate. The method was later developed by Oerum (1905). Autenrieth (1910) used the method for the determination of urinary indican.

The Condensation of Isatin and Indoxyl.

The investigation of indole metabolism necessitated the estimation of urinary indican, and an examination was made of the suitability of the isatin condensation for indican estimation. Previous workers in this field hydrolysed indican with hydrochloric acid, and consequently this acid was used in the early stages of the investigation. A considerable variation in the results was apparent from the outset; estimations made under identical conditions on the same sample of urine differed by as much as 40%. No consistency could be observed in this variation, other than that the lowest results were obtained after prolonged heating during hydrolysis; variation in the concentration of isatin and acid yielded no information. Ignorance of the true indican values rendered evaluation difficult. After some difficulty, a specimen of pure potassium indoxyl sulphate was obtained commercially, from



The dotted line shows the theoretical value.

which the standard was prepared. The indoxyl content of this standard solution being known, the deviation of the result obtained from the theoretical value was

also known. Experiment showed that the inconsistency was due in part to prolonged heating during hydrolysis. The curve shows the effect of continued heating during hydrolysis: eight tubes each containing 5 ml. of a standard indoxyl solution (1 ml. = .05 mg.), 2 ml. of a saturated solution of isatin in water, and 2 ml. of conc. HCl were placed in a boiling water bath, a tube was removed every ten minutes, and the indirubin extracted and estimated photometrically. These results indicate the destruction of the indirubin on prolonged heating. It will be observed that the variation produced by prolonged heating is irregular, and is not proportional to the duration of the heating period. Reduction of the heating period to a minimum by bringing the tubes to boil in a flame and allowing to cool reduced the error as shown by the following six results of estimations made on solutions each containing 0.25 mg. indoxyl sulphate.

0.23 0.23 0.24 0.24 0.23 0.23

It will be seen that the error, whilst reduced and more consistent, is still present. The most likely cause of the error is hydrochloric acid, and it was decided to investigate the effects of replacement of hydrochloric by sulphuric acid as the hydrolysing agent. The acid was found to yield very satisfactory results. Standard solutions of indican gave theoretical results after 15 minutes immersion in a boiling-water bath. The following table shows the results of estimations on a series of standard indican solutions.

PRESENT	FOUND.
mg. indican	mg. indican
0.05	0.05
0.15	0.14
0.25	0.25
0.35	0.35
0.40	0.39
0.45	0.45
0.50	0.49

The method was found to give equally satisfactory results with urine.

The following are the results of estimations on a sample of normal urine. Except where otherwise stated the solutions were estimated as described in Methods of Analysis.

	URINE	INDICAN FOUND. Mg.
1.	2.5 ml.	0.48
2.	2.5 ml	0.48
3.	2.5 ml.	0.48
4.	2.5 ml. + 10 mins. in bath	0.48
5.	2.5 ml. + 15 ,, ,, ,, ...	0.48
6.	2.5 ml. + 20 ,, ,, ,, ...	0.48

URINE	INDICAN FOUND. Mg.
7. 5.0 ml.	0.96
8. 2.5 ml. + neutral Pb Acetate ...	0.48
9. 2.5 ml. + neutral Pb Acetate ...	0.48
10. 2.5 ml. + basic Pb Acetate	0.35
11. 2.5 ml. + basic Pb Acetate	0.30

Solution 8 was prepared by adding 2 ml. of neutral lead acetate to 20 ml. of urine and filtering: 2.75 ml. ≡ 2.5 ml. original urine. Solution 9 by adding 4 ml. neutral acetate to 20 ml. of urine: 3 ml. ≡ 2.5 ml. of original urine. Solutions 10 and 11 were prepared in a similar manner to 8 and 9, using basic lead acetate in place of neutral lead acetate.

It is affirmed by some workers, and denied by others, that the clarification of urine with lead acetate causes loss of indican. This conflict of result is probably due to the use of different forms of lead precipitant. It will be seen from the above table that the two urines clarified with neutral lead acetate (8, 9) have not undergone any loss, whilst those clarified with basic lead acetate have (10, 11). Indican is partially precipitated by lead in alkaline solution, and this property has been utilised for its isolation (Neuberg and Schwenk, 1917; Rothman, 1944). H. Eucker (1938) has reported considerable loss of indican on clarifying urine with $Pb(OH)_2$. The discrepancies therefore, are presumably due to the use of basic lead acetate as a clarifying agent by some workers, consequently the use of this reagent should be avoided in indican estimations.

Procedure.

Reagents. Isatin Solution. A saturated solution of pure isatin in distilled water, which is a concentration of approximately 0.15%.

Standard Indirubin Solution. Prepared by dissolving 0.05 g. of pure indirubin in 500 ml. of chloroform: 1 ml. = 0.1 mg. Pure indirubin may be obtained commercially, or may be synthesized by running a solution of indoxyl, prepared from phenyl glycine o-carboxylic acid (Cumming, Hopper and Wheeler, 1923), into an acid solution of isatin, and purifying the indirubin by sublimation. The presence of indigo may be detected by absorption on to a column of alumina from $CHCl_3$ solution.

Standard Indoxyl Solution. The standard indoxyl solution used in the investigation was prepared by dissolving 0.05 g. of potassium indoxyl sulphate ' Schuchardt ' in 100 ml. of distilled H_2O. This solution will keep more than one month in the dark.

Sulphuric Acid. 50% sulphuric acid.

Sodium Hydroxide. 20% sodium hydroxide.

Method of Analysis. The sample of urine should be preserved under toluene. Thymol must not be used as a preservative as it forms a coloured compound with indoxyl in acid solution. The indican value of urine begins to decrease after 24 hours. Several workers recommend precipitation of the urine with lead acetate to render subsequent extraction easier. This was found to be unnecessary; if the extracts with chloroform tend to emulsify they may be cleared rapidly by centrifuging.

2.5 ml. of the urine are measured into a test tube, 2 ml. of saturated isatin solution are added, followed by 1 ml. of 50% H_2SO_4. The solution is brought to boil over a flame, and then placed in a boiling-water bath for 5 minutes, removed, and allowed to cool. The solution is washed into a separating-funnel and extracted with two 5 ml. portions of $CHCl_3$. Care should be taken to see that extraction is complete. The resultant extract, which contains both indirubin and isatin, is transferred to the separating-funnel, an equal volume of water added, and five drops of 20% NaOH added.

The action of the alkali converts the isatin to isatic acid by ring opening, and this dissolves in the aqueous layer. The chloroform solution of indirubin is run off into a graduated centrifuge tube, clarified by centrifuging if necessary, and made up to 10 ml. and estimated by comparison with standards or in an absorptiometer. Throughout this investigation a Spekker absorptiometer was used for estimation. Indirubin values may be converted into indoxyl values or *vice versa* by the relationship,

1 gram of potassium indoxyl sulphate = 1.04 grams indirubin.

The minimum quantity of indican which can be detected and estimated by this method is of the order of 4 micrograms.

SUMMARY.

A colorimetric method is described for the estimation of urinary indoxyl, based on the condensation of isatin and indoxyl to form indirubin.

This investigation was undertaken at the suggestion and under the supervision of Dr. W. R. Fearon, in the Department of Biochemistry, Trinity College, Dublin, and was made possible by a grant from the Medical Research Council of Ireland.

I am indebted to Dr. T. S. Wheeler for a quantity of o-amino benzoic acid.

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